Kidney International, Vol. 37 (1990), pp. 1301–1309

Intra- and extracellular proteins in human normal and polycystic kidney epithelial cells

YOSEF GRANOT, VICKI VAN PUTTEN, JULIE PRZEKWAS, PATRICIA A. GABOW, and ROBERT W. SCHRIER

Department of Medicine, University of Colorado School of Medicine, Denver, Colorado, USA

Intra- and extracellular proteins in human normal and polycystic kidney epithelial cells. A tissue culture method was established for the continuous growth of epithelial cells from the cortex of human normal kidney (HNC) and from the epithelial layer of kidney cysts from autosomal dominant polycystic kidney disease (ADPKD) patients. Primary cells were grown to 80 to 90% confluency from 1 mm^2 slices of tissue, and subcultured up to 10 times. The subcultured HNC and ADPKD cells retained characteristic epithelial polygonal and elongated shape and positive immunofluorescent staining for cytokeratin. The cell doubling time for both HNC and ADPKD epithelia was three to four days at a fetal calf serum (FCS) concentration of 5%. Using these culturing procedures 1 to 5×10^9 epithelial cells could be obtained from each kidney specimen. Profiles of ^{35}S-methionine radiolabeled intracellular proteins of HNC and ADPKD cells qualitatively demonstrated a high degree of similarity, thus confirming a similarity of epithelial origin and protein biosynthesis. Both the underexpression of three proteins (a) protein p2, $M_r \sim 47$ kDa, pI ~ 6.0; b) protein p3, $M_r \sim 50$ kDa, pI ~ 5.9; and c) protein p4, $M_r \sim 44$ kDa, pI ~ 5.8) and the overexpression of several proteins (including: a) p5, $M_r \sim 56$ kDa, pI ~ 7.3; b) protein p6, $M_r \sim 32$ kDa, pI ~ 7.3; c) protein p7, $M_r \sim 33$ kDa, pI ~ 5.3; d) protein p8, $M_r \sim 45$ kDa, pI ~ 6.9; e) protein p9, $M_r \sim 35$ kDa, pI ~ 6.7; and f) protein p10, $M_r \sim 30$ kDa, pI ~ 6.6) were found in ADPKD cells. In addition, primary ADPKD cells, but not primary HNC cells, produced three extracellular proteins: $M_r \sim 220$, 170 and 45 kDa. Upon subculturing, identical extracellular proteins were biosynthesized by both HNC and ADPKD cells. This subculturing system provides an important methodological tool for the study of the molecular and biochemical abnormalities of ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD) is an important hereditary cause of end-stage renal failure [1–3] and accounts for 10 to 12% of all end-stage renal disease (ESRD) in this country [4]. Present data suggest that the pathogenesis of polycystic kidney disease (PKD) involves alterations in both basement membrane structure [5, 6] and epithelial cell growth [7–9]. Electron microscopy studies have provided evidence for abnormal basement membranes in both ADPKD kidneys [10–12] and rat kidneys with toxin induced PKD [5, 6]. In addition, the systemic nature of PKD strongly suggests a pathogenic scheme involving cell-basement membrane interactions [13]. The observation of increased epithelial cell number in ADPKD [7–9] supports the concept of altered tubular growth in this disease. In addition, the observation of a variety of polypoid lesions within renal cyst walls in human and experimental models of PKD supports uncontrolled growth. The increased incidence of tumors in certain forms of PKD [14–16], as well as the elevated c-myc gene expression in a mouse model of PKD [17] provide additional support for the possible involvement of uncontrolled cell proliferation in the pathogenesis of PKD.

A major advance in the identification of the genetic lesion in ADPKD has been the localization of one of the putative genes to human chromosome 16 [18–20]. Recently, several ADPKD families have been identified that develop ADPKD that is not genetically linked to chromosome 16 [21–24]. Unfortunately, the molecular and biochemical expression of the ADPKD genetic defect which leads to the formation of cysts is still unknown. The development of an epithelial cell tissue culture system from human cysts can aid in the assessment of the molecular and biochemical events involved in cyst formation. To this end, a method for culturing primary epithelial cells from ADPKD cysts was recently developed [25]. However, the limited number of cells obtained by this procedure does not permit the isolation of sufficient quantities of DNA, mRNA(s) and possible genetically-modified proteins related to ADPKD needed for this type of study.

In this report, we describe the establishment of a tissue culture system from normal human kidney cortex (HNC) and the epithelial layer of ADPKD cysts which provides 1 to 5×10^9 epithelial cells from one kidney specimen. In addition, we have characterized the profiles of the intra- and extracellular proteins biosynthesized in these cultures. The production of such large numbers of HNC and ADPKD epithelial cells in a continuous cell culture will facilitate research into the genetically oriented biochemical and molecular events associated with the ADPKD disorder.

Methods

Fluorescein conjugated goat anti-mouse IgG antibody, bovine serum albumin (BSA) and ^{35}S-methionine were purchased from ICN Biochemicals (Cleveland, Ohio, USA); epidermal growth factor (EGF), dexamethasone and transferrin were from Sigma Chemical Co. (St. Louis, Missouri, USA), insulin, penicillin and streptomycin from Gibco Laboratories (Grand Island, New York, USA), ^{3}H-thymidine from Dupont NEN

Received for publication June 26, 1989
and in revised form November 6, 1989
Accepted for publication December 18, 1989

(Wilmington, Delaware, USA). Balanced Hanks salt solution (BSS), Roswell Park Memorial Institute Medium (RPMI-1640), and fetal calf serum (FCS) were from Hazelton; and NTB2 emulsion, D19 developer and EKTAflow were from Kodak (Eastman Kodak, Rochester, New York, USA). Monoclonal anticytokeratin antibody was supplied by R. Cardiff, University of California, Davis (California, USA). Cadaveric human kidneys not suitable for transplantation were obtained through the National Disease Research Interchange (Philadelphia, Pennsylvania, USA). ADPKD kidneys were obtained from patients with ESRD who underwent kidney transplantation. Several renal transplant centers have donated the surgical and autopsy specimen ADPKD kidneys.

Primary ADPKD cells

The preparation of primary epithelial cells was performed according to the procedure described by Wilson et al [25]. Approximately 10 ADPKD cysts were individually dissected from each ADPKD kidney specimen. Each cyst was stripped of any fibrous tissue, cut open and washed five times in human epithelia dissection medium (HED, RPMI-1640, pH 7.2 containing 5 μg/ml human transferrin, 10 mM 4-12-(hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 24 mM $NaHCO_3$, 150 U/ml penicillin and 150 μg/ml streptomycin). The epithelial lining of the cyst was stripped away under 12 × magnification and chopped into 1 mm^2 pieces using a scalpel. The pieces were explanted onto a collagen-coated gas-permeable 60 mm Teflon membrane culture dish in 0.7 ml human epithelia primary growth medium (HPG, RPMI-1640, pH 7.2 containing 5 μg/ml human transferrin, 10 mM HEPES, 24 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin plus 4 ng/ml dexamethasone, 50 ng/ml insulin, 3.3 nM EGF, and 3% FCS). Approximately 30 pieces were explanted per 60 mm dish. All tissue culture was performed at 37°C, 94% humidity and 5% CO_2. After 18 hours the explanted pieces were fed with an additional 0.7 ml of HPG media. Cultures were fed every two to three days. After the cells in the primary cultures reached 80 to 90% confluency (7 to 10 days) the pieces were reexplanted to new collagen-coated 60 mm Teflon membrane culture dishes for the start of a new primary culture. It was possible to re-explant these pieces at least four additional times.

Subcultured ADPKD cells

ADPKD cells were subcultured at 80 to 90% confluency. HPG was aspirated and replaced with 2.5 ml of a solution containing 0.25% trypsin and 0.1% EDTA-Na_2 in BSS (pH 7.4, 5.4 mM KCl, 0.4 mM KH_2PO_4, 137 mM NaCl, 4 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 5.5 mM glucose). The cells were incubated at 37°C for 10 minutes. At this time four volumes of human epithelia subculturing growth medium (HSG, RPMI-1640, pH 7.2 containing 5 μg/ml human transferrin, 10 mM HEPES, 24 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 ng/ml dexamethasone, 50 ng/ml insulin, 3.3 nM EGF, and 5% FCS) was added. The cells were then aspirated from the culture dishes, centrifuged for two minutes (150 × g), re-suspended in HSG and divided into new 100 mm plastic culture dishes containing the same medium at cell concentrations of 10^6 cells/dish. After the cells had attached (approximately 16 hr), the medium was aspirated and the cells fed with 8 ml HSG.

HNC cells

Primary cultures of human cortical kidney pieces were grown under the same conditions described for ADPKD cells. One mm^2 pieces of the cortical region of the human kidneys were prepared and washed in HED. Approximately 30 pieces were explanted onto 60 mm collagen-coated Teflon membrane culture dishes. These primary cultures were grown in HPG. The pieces were then re-explanted to new 60 mm Teflon membrane culture dishes for the start of new primary cultures. The primary cultured cells were subcultured by the method described for the ADPKD cells. All ADPKD and HNC cultures were used experimentally after 7 to 14 days of growth as assessed by cell number.

Indirect immunofluorescence for cytokeratin

The epithelial nature of the ADPKD and HNC cells was ascertained by immunofluorescent staining for cytokeratin. Cytokeratins are widely regarded as markers for epithelial differentiation [26]. A monoclonal anticytokeratin antibody was used to localize the cytokeratin filaments. This antibody was prepared against a human breast carcinoma and was shown not to cross react with vimentin [27]. The cells were fixed in 30% methanol/70% acetone (vol/vol) at −20°C for 10 minutes, washed three times with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$), and exposed to the primary antibody for 30 minutes. Following several rinses in PBS the cells were stained with a fluorescein conjugated goat anti-mouse IgG antibody and the percentage of cells exhibiting filament staining quantitated by fluorescence microscopy. Cells stained only with the fluorescein conjugated antibodies served as negative controls.

Quantification of HNC and ADPKD cell growth

Cell number was determined by the counting of trypsinized cells using a Bright Line hemacytometer. Cells were plated with an initial density of 150,000 cells/60 mm culture dish and counted on days 1, 2, 4, 6, and 8 for the growth experiments.

^{3}H-thymidine radiolabeling of ADPKD cell nuclei

The rate of cell growth was assessed by measurement of ^{3}H-thymidine incorporation in ADPKD cell nuclei. Briefly, cells were incubated with 0.2 to 0.5 μCi/ml ^{3}H-thymidine in HSG with or without 3.3 nM EGF and with varying amounts of FCS for 24 hours. At this time the cells were washed five times with saline and 0.1% albumin, fixed with methanol for 10 seconds and air dried. In a dark room the culture dishes were coated with Kodak NTB2 emulsion and dried for two hours. The dishes were then placed in a light-tight box at 4°C for three to four days. The cells were developed with Kodak D19 developer for four minutes, 1% acetic acid for one minute and fixed with Kodak EKTAflow for five minutes. The dishes were washed under cold running tap water for 10 minutes, air dried and stained with 0.1% toluidine blue. The cells were subsequently examined at 100 × magnification and the number of nuclei stained were counted. Percent incorporation was determined by (number of stained nuclei)/(total number of cells present).

Radiolabeling of cell proteins with ^{35}S-methionine

When the various cell cultures were 80 to 90% confluent, the proteins were radiolabeled by incubation with 75 μCi/ml ^{35}S-methionine (700 Ci/mmol) at 37°C for 24 hours in the appropriate growth media without methionine. The medium was collected for the identification of biosynthesized extracellular proteins and stored at −70°C. The cells were then washed two times with cold (4°C) Tris buffered saline (STE, 150 mM NaCl, 20 mM Tris, pH 7.2, containing 1 mM EDTA-Na_2), scraped from the dishes and pelleted by centrifugation. The pellets were quick frozen in liquid N_2 and stored at −70°C for future identification of the biosynthetically radiolabeled intracellular proteins.

Electrophoretic separation of radiolabeled proteins

Radiolabeled proteins from the extracellular media or from the fresh or frozen cells were resolved according to specific M_rs using one dimensional (1D) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and the buffer system described by Laemmli [28]. The 12 × 18 cm slab gels were 0.75 mm thick and composed of 10 or 12.5% acrylamide and 0.2 or 0.25% bis-acrylamide. A more definitive resolution of the HNC and ADPKD proteins was achieved with the two dimensional (2D) electrophoresis system described by O'Farrel [29]. The first dimensional separation was produced by isoelectric focusing of the proteins according to their specific isoelectric points (pI) on gels with a pre-established pH 5.0 to 7.5 gradient (1.6% ampholines, pH 5 to 8; 0.4% ampholines, pH 3 to 10). The second dimensional separation was achieved using the SDS-PAGE system. Following the electrophoretic separation of the proteins the gels were stained with coomassie blue and dried onto Whatman 3 MM paper. Molecular weights were determined by comparison with commercial standards. The ^{35}S-methionine radiolabeled proteins were detected by autoradiography as has been described [30]. Densitometric quantification was performed on a 300A Computing Densitometer from Molecular Dynamics.

Due to the sporadic availability of both normal and ADPKD kidneys, the HNC and ADPKD cell cultures were radiolabeled as the cells became available. To ensure that the autoradiograms could be directly compared, the amount of radioactivity loaded per sample was kept constant (5 × 10^5 cpm). This corrected for the half-life decay of the ^{35}S-methionine radiolabeled proteins, kept the radiolabeled proteins in proportional quantities and resulted in 0.9 to 3 μg protein per sample.

Protein determinations

Protein determinations were made using a modification of the Lowry et al method [31].

Results

Cell culture of ADPKD epithelia

Recently, a method was established for the primary culture of epithelial cells from the lining of ADPKD kidney cysts [25]. Using this method, we have obtained primary cultures from the cysts of 12 ADPKD kidneys which reached 80 to 90% confluency on 60 mm collagen-coated Teflon membrane culture dishes. These cells are pictured in Figure 1A after 14 days of growth (100 ×). After the primary cultures reached 80 to 90% confluency, it was possible to subculture the ADPKD cells by trypsinization in 100 mm plastic culture dishes (10^6 cells/dish) to 80 to 90% confluency (5 × 10^6) cells within 7 to 10 days. In Figure 1B a fifth ADPKD cell passage is shown (100 ×). All 12 ADPKD primary tissue cultures were subcultured up to 10 passages.

Virtually all the cells in the cultures were determined to be epithelial as evidenced by positive immunofluorescent staining for cytokeratin (Fig. 1C).

Cell culture of normal kidney, human cortex epithelial cells

Using the same methods described above for the development of an ADPKD epithelial culture system, a system for primary (Fig. 2A) and subcultured (Fig. 2B) HNC cells from eight different kidneys was established. These cells were also characterized by positive immunostaining for cytokeratin (Fig. 2C). In addition, when a frozen section of normal human renal cortex was examined, only the cells of tubular epithelial origin exhibited specific immunofluorescent staining for cytokeratin (Fig. 2D).

Quantification of HNC and ADPKD epithelial cell growth

Recently, the kidney has been identified as one of the major sites of EGF production [32]. In addition, previous studies have shown that EGF (1 to 15 nM) increases ^{3}H-thymidine incorporation in cultured normal human glomerular mesangial cells 10- to 12-fold [33]. Therefore, to assess the requirement of EGF for ADPKD cell growth, ^{3}H-thymidine incorporation into ADPKD cell nuclei was measured. This was done in the absence or presence of 3.3 mM EGF at varying FCS concentrations. EGF significantly increased ^{3}H-thymidine incorporation as shown in Figure 3 (with 5% FCS, 63.5 ± 4.5% without EGF vs. 78 ± 1% of EGF, $P < 0.05$). Thus, EGF was routinely added to the growth media.

The time course of HNC or ADPKD epithelial cell growth is shown in Figure 4. The rate of cell growth was estimated by cell number in 60 mm culture dishes on days 1 to 8. The doubling time for both HNC and ADPKD epithelial cells was approximately three to four days. As the number of passages increased (higher than 5) there was a substantial reduction in cell multiplication. Therefore, the cells used in the experiments described were from cell passages 2 to 5.

The effect of the FCS concentration in the growth medium on subcultured HNC and ADPKD epithelial cell growth following 10 days of proliferation is presented in Figure 5. Under these conditions the optimal FCS concentration for the growth of both subcultures appeared to be 5%. Thus, both cell types had similar requirements for FCS and growth factor supplements in the media.

The multiple passages of the HNC and ADPKD epithelial pieces as well as the subculture of these cells can potentially produce 1 to 5 × 10^9 epithelial cells from one normal or one ADPKD human kidney specimen.

^{35}S-methionine protein profiles in HNC and ADPKD epithelia

The protein profiles of HNC and ADPKD epithelia were examined by ^{35}S-methionine protein radiolabeling and SDS-PAGE separation. The profile of biosynthetically radiolabeled intra- and extracellular proteins from primary HNC and ADPKD epithelial cells is shown in Figure 6. As illustrated in

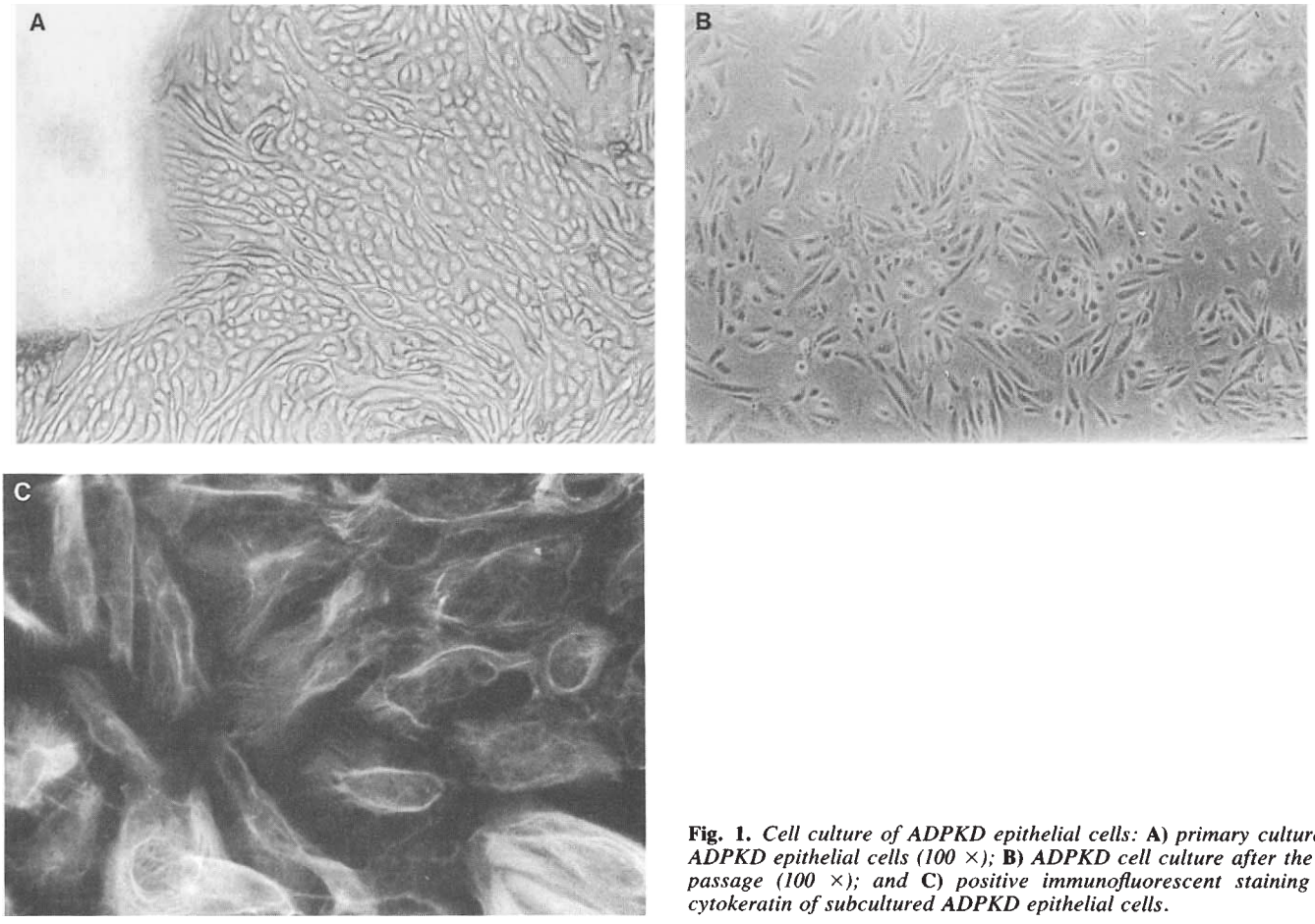

Fig. 1. *Cell culture of ADPKD epithelial cells:* **A)** *primary culture of ADPKD epithelial cells (100 ×);* **B)** *ADPKD cell culture after the 5th passage (100 ×); and* **C)** *positive immunofluorescent staining for cytokeratin of subcultured ADPKD epithelial cells.*

this Figure (lanes A and C), only the ADPKD primary cells produced extracellular radiolabeled proteins with apparent M_r of 220, 170 and 45 kDa. On the other hand, there is a significant similarity in the electrophoretic separation of the ^{35}S-methionine radiolabeled intracellular proteins from the HNC and ADPKD epithelial cells (lanes B and D). It is also clear that in the apparent M_r 44 kDa there is an underexpression of ^{35}S-methionine radiolabeled proteins in ADPKD cells.

Two-dimensional electrophoresis [29] was used to better define the similarities and differences in the HNC and ADPKD epithelial cell intracellular protein profiles. In Figure 7 is illustrated the resolution of at least 465 radiolabeled proteins biosynthesized in HNC (left) or ADPKD (right) cells. The comparison of the protein spots demonstrated a high degree of similarity in these protein profiles. Interestingly, the most obvious differences that appear in ADPKD cells are: 1) The underexpression of three proteins: a) protein p2, $M_r \sim 47$ kDa, pI ~ 6.0; b) protein p3, $M_r \sim 50$ kDa, pI ~ 5.9; and c) protein p4, $M_r \sim 44$ kDa, pI ~ 5.8. 2) The overexpression of several proteins including: a) protein p5, $M_r \sim 56$ kDa, pI ~ 7.3; b) protein p6, $M_r \sim 32$ kDa, pI ~ 7.3; c) protein p7, $M_r \sim 33$ kDa, pI ~ 5.3; d) protein p8, $M_r \sim 45$ kDa, pI ~ 6.9; e) protein p9, $M_r \sim 35$ kDa, pI ~ 6.7; and f) protein p10, $M_r \sim 30$ kDa, pI ~ 6.6. Densitometric quantification of the differences in these proteins is presented in Table 1. Similar observations were obtained in tissue cultures from two different HNC and ADPKD kidney specimens (data not shown).

The time course for the ^{35}S-methionine radiolabeled extracellular proteins in ADPKD epithelial cells with and without FCS is pictured in Figure 8. As shown in this figure, biosynthesis of the 220, 170 and 45 kDa extracellular proteins is time dependent. Densitometric quantification revealed a tenfold increase between 3 and 24 hr. In addition, 5% FCS further increases this biosynthesis by three- to fourfold at each indicated time. The intracellular protein biosynthesis following 24 hour exposure to ^{35}S-methionine was also stimulated two- to threefold by the presence of 5% FCS in the incubation medium. The similarity of the intra- and extracellular protein profiles of ADPKD cultures from four patients (A through D) is presented in Figure 9. Similar protein profiles have been observed in five additional patients (data not shown).

In Figure 10 are depicted experiments performed to answer the question as to whether the biosynthesis of the 220, 170 and 45 kDa extracellular proteins is a unique phenomenon for ADPKD epithelial cells that is conserved throughout the subculturing process. Comparison studies of intra- and extracellular proteins in primary and subcultured epithelial cells from one HNC (Fig. 10A) or ADPKD (Fig. 10B) specimen were conducted. In ADPKD cell cultures (Fig. 10B) both intra- and extracellular protein profiles retained a high degree of similarity

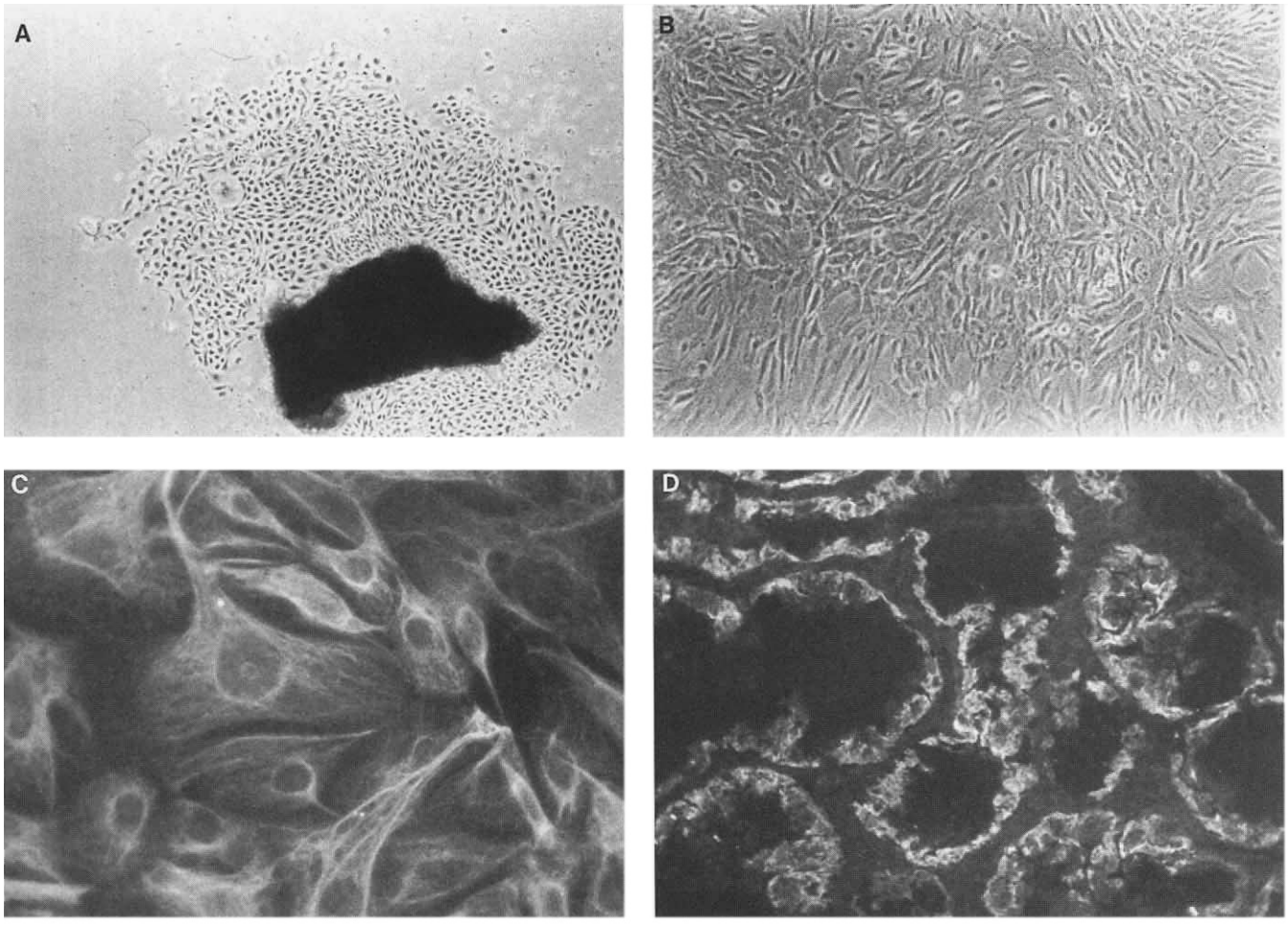

Fig. 2. *Cell culture of HNC epithelial cells:* **A)** *primary culture of HNC epithelial cells (40 ×);* **B)** *HNC epithelial cell culture after the 5th passage (100 ×);* **C)** *positive immunofluorescent staining for cytokeratin of subcultured HNC epithelial cells; and* **D)** *positive immunofluorescent staining for cytokeratin of the tubular epithelia in a frozen section of normal human kidney cortex.*

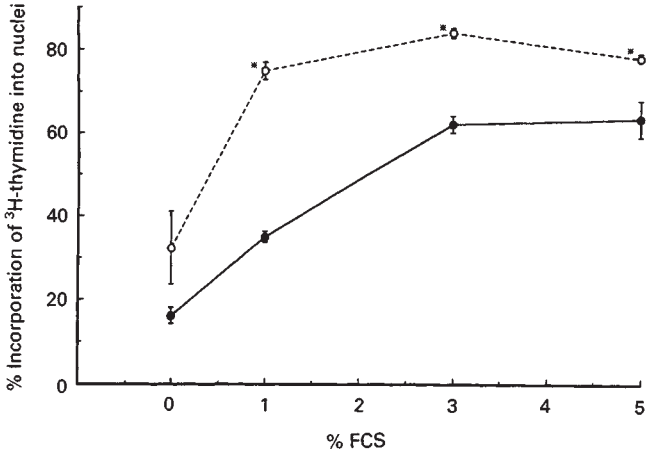

Fig. 3. *^{3}H-thymidine incorporation into ADPKD cell nuclei.* The incorporation of ^{3}H-thymidine into ADPKD cell nuclei was measured, as described in the **Methods**, in the absence or presence of 3.3 mM EGF with varying concentrations of FCS (0, 1, 3 and 5%). (mean ± SE, closed circles, 0 mM EGF, $N = 2$; open circles, 3.3 mM EGF, $N = 2$)

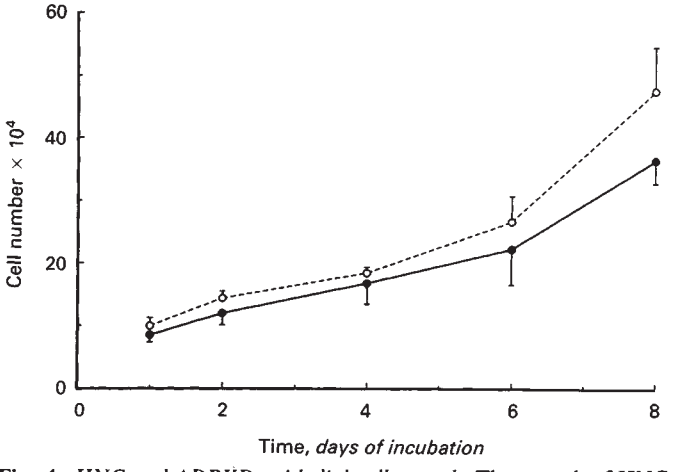

Fig. 4. *HNC and ADPKD epithelial cell growth.* The growth of HNC and ADPKD epithelial cells (passages 1–5) was initiated by distributing 15×10^4 cells per 60 mm culture dishes on day zero. The cells were then incubated in medium with 5% FCS. On the days indicated, the medium was aspirated, the cells trypsinized, removed from the dishes and counted. The cell numbers presented are the mean ± SE of 9 and 7 different HNC and ADPKD specimens, respectively. (Closed circles, HNC epithelial cells; open circles, ADPKD epithelial cells)

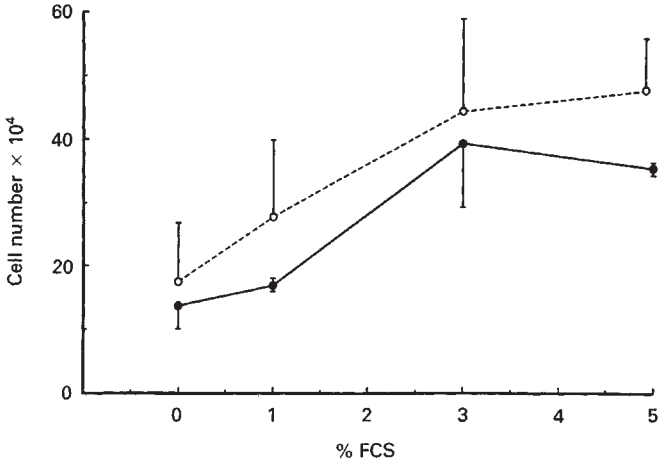

Fig. 5. *The effect of increasing concentrations of FCS on HNC and ADPKD cell growth.* The growth of the HNC and ADPKD cells was initiated as described in Figure 3 and the cells were grown for 10 days in media containing 0 to 5% FCS concentrations. The results represent the mean ± SE for 3 HNC and 5 ADPKD cultures. (Closed circles, HNC epithelial cells; open circles, ADPKD epithelial cells)

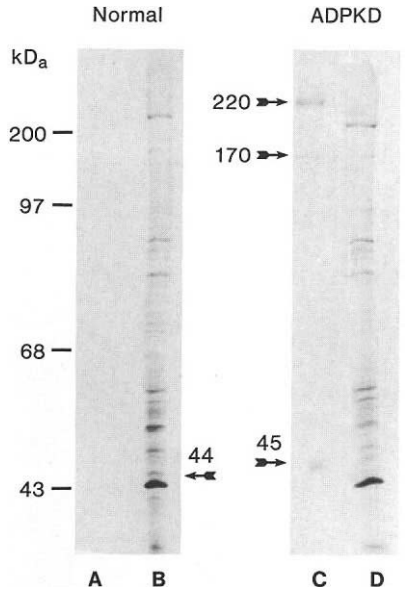

Fig. 6. *Intra- and extracellular proteins in HNC and ADPKD primary cultures.* The ^{35}S-methionine radiolabeled proteins from HNC and ADPKD epithelial cells were prepared and separated by 10% SDS-PAGE as described in **Methods**. (Lane A, HNC extracellular proteins; lane B, HNC intracellular proteins; lane C, extracellular ADPKD proteins; lane D, ADPKD intracellular proteins)

with the three extracellular proteins (apparent M_r ~ 220, 170 and 45 kDa) evident in all cultures (primary, 1, 2, 3 and 5, Lanes A through E, respectively). The intracellular protein profiles of HNC cells retained a high degree of similarity throughout the passages. However, the extracellular proteins (220, 170 and 45 kDa) were only observed after the cells were subcultured. The three extracellular proteins were present in HNC epithelial cells in relatively large quantities after 2, 3 and 5 passages. A comparison of these three extracellular proteins derived from both HNC and ADPKD cells by 2D gel electrophoresis revealed a high degree of similarity in their M_rs and pIs (data not shown).

Discussion

ADPKD is the most common hereditary renal disease, affecting 500,000 Americans. Little is known, however, about the pathogenesis of ADPKD, in part because there is not a naturally occurring autosomal dominant animal model with which to study this disease. Thus, there exists a lack of readily available tissue necessary to examine the cellular and molecular pathogenetic events of ADPKD. The establishment of a primary culture of ADPKD cyst epithelial cells [25] was a major step toward an understanding of the pathogenesis of this disorder. However, primary cultures cannot supply the volume of cells needed for biochemical and molecular studies, and such cultures are dependent on the frequent and continuous availability of kidneys from ADPKD and normal patients.

In this report we have described a continuous tissue subculture system for ADPKD (Fig. 1) and HNC (Fig. 2) epithelial cells from 12 ADPKD and 8 normal human kidneys. These cells can be subcultured up to 10 times. With the growth rate of both HNC and ADPKD epithelial cells (Fig. 4), the use of FCS for increased cell growth (Fig. 5), and the use of subculturing procedures in both tissue culture systems, it is possible to obtain approximately 1 to 5 × 10^9 cells from each ADPKD or normal kidney specimen. The main advantage of this approach is that an initial specimen from a single normal or ADPKD kidney will generate sufficient HNC and ADPKD epithelial cells to allow extensive biochemical and molecular biology studies.

A subcultured tissue culture system is valid only if the cellular characteristics of the primary culture can be reproduced throughout the passages. Of critical importance, therefore, was the demonstration that all the cultures maintained positive immunofluorescence for cytokeratin, (Figs. 1 and 2), a characteristic of epithelial cells. This finding indicated that the tissue culture system remained free from harmful fibroblast overgrowth. Fibroblast contamination would make it impossible to delineate the specific characteristics of ADPKD and HNC epithelial cells.

It has been reported in a previous study from this laboratory [25] that the primary cell growth and multiplication of ADPKD epithelial cells is much faster than that of primary epithelial cells obtained from different segments of the normal human kidney. In this report we have demonstrated that cell growth and multiplication of subcultured cells of both HNC and ADPKD epithelia (passages 1 to 5, Fig. 4) induced by 5% FCS, insulin, dexamethasone, transferrin and EGF is similar (doubling time 3 to 4 days). In addition, insulin, dexamethasone, transferrin, and EGF in combination with different FCS concentrations (0 to 5%) did not modify this similarity in multiplication time (Fig. 5). These data indicate that there is no differential effect of FCS or the growth factors on the growth rate of the HNC or ADPKD epithelial cell types.

Under the conditions described in this study the doubling time of both the HNC and ADPKD subcultured cells is similar, yet ADPKD kidneys demonstrate an obvious increase in cell growth in vivo as evidenced by enlarged kidney size and increased cell number within cyst walls [7–9]. Subtle changes in the rate of cell growth which could, over a lifetime, result in

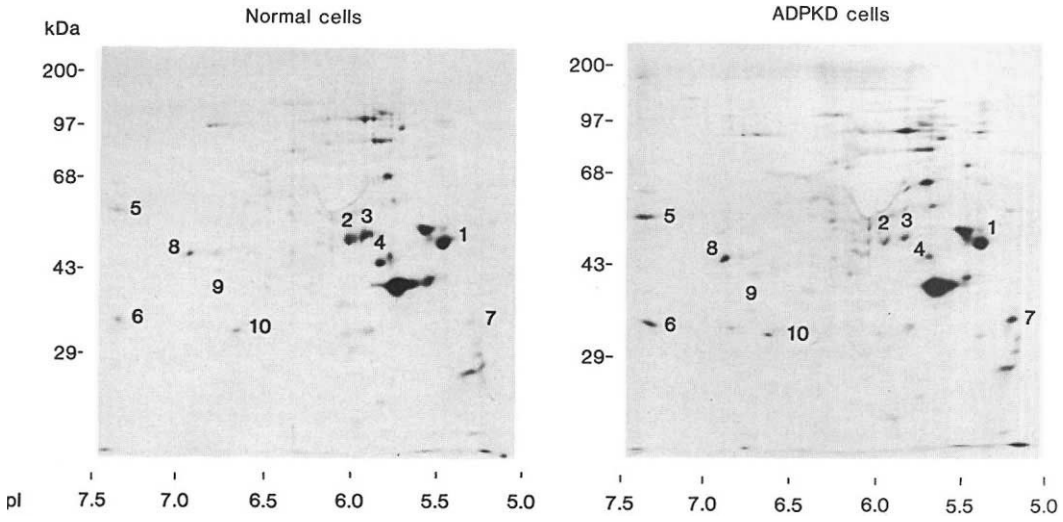

Fig. 7. *Two dimensional separation of intracellular proteins from HNC* (**A**) *and ADPKD* (**B**) *epithelial cells.* The ^{35}S-methionine radiolabeled intracellular proteins were separated by 2D gel electrophoresis (10% SDS-PAGE) [26]. The numbers indicate the proteins in HNC and ADPKD cells that were quantitated by densitometry. (kDa, relative molecular weight of the proteins; pI, isoelectric points across the gels)

Table 1. Densitometric quantification of the proteins identified in Figure 7

Protein	kDa	pI	HNC *Relative density*	%	ADPKD *Relative density*	%
p1	47	5.4	4529	100	4419	100
p2	47	6.0	2161	47.7	1092	24.7
p3	50	5.9	2331	51.4	1021	23.1
p4	44	5.8	1568	34.6	125	2.8
p5	56	7.3	415	9.1	1569	35.5
p6	32	7.3	424	9.4	1375	31.1
p7	33	5.3	215	4.7	1397	31.6
p8	45	6.9	456	10.1	1268	28.6
p9	35	6.7	30	0.7	109	2.5
p10	30	6.6	21	0.5	641	14.5

The values in the % column are a standardization of the relative densities and expressed as a % of the relative density of p1. This protein has the same relative density in both HNC and ADPKD cells.

increased cell number in ADPKD kidneys may not be detectable by the described methods used to measure these growth rates in cell culture. Therefore, the measured growth characteristics of these cultured cells must not represent the actual growth potential of human cystic or normal kidney cells during a person's lifetime.

To further verify the retention of cellular characteristics in these two tissue culture systems we examined intra- and extracellular protein biosynthesis in both HNC and ADPKD tissue cultures (Figs. 6 and 7). The obvious similarity in the profiles of the intracellular biosynthetically radiolabeled proteins of the HNC and ADPKD cells (Figs. 6 and 7) throughout the passages (Fig. 10) demonstrates the close relationship of these cells. Therefore, the genetic information translated into cell proteins is preserved in the HNC and ADPKD epithelial subculturing procedure. Because of this conservation we were able to systematically examine more than 465 proteins with regard to their relative molecular mass and isoelectric points in both HNC and ADPKD epithelia (Fig. 7). Thus, these cultures (primary through 1 to 5 passages) are eminently suitable for biochemical and molecular studies of protein biosynthesis.

Carone et al [13] have previously reported abnormalities in protein biosynthesis and secretion by both intra- and extracellular proteins in the rat model of 2-amino-4,5-diphenyl thiazole-induced renal cystic disease. The present study demonstrates the biosynthesis of three extracellular proteins by human ADPKD cells. These proteins, however, begin to appear in later passages of HNC cultures (Fig. 10). Two dimensional electrophoresis shows that the extracellular ADPKD proteins and the extracellular HNC proteins have a high degree of similarity (data not shown). Thus, indicating it is probable that the defective ADPKD gene does not directly code for these proteins. It is possible that this protein biosynthesis is, instead, a secondary effect of the ADPKD defect and subculturing induces an artifactual release of these proteins in HNC normal cells.

Abnormal protein synthesis is also evident within the intracellular pool of proteins. Neither the over- nor underexpression of intracellular ^{35}S-methionine radiolabeled proteins in ADPKD epithelial cells (Figs. 6 and 7) are affected by subculturing and the abnormalities remain throughout all passages (Fig. 10). The differences seen between ADPKD and HNC cells (Fig. 7) may

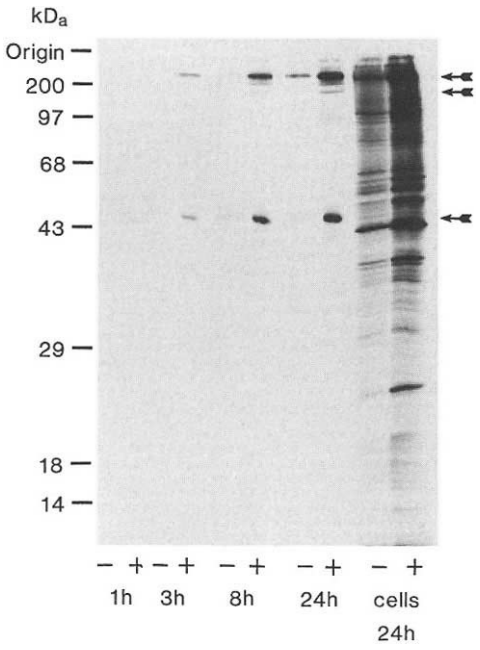

Fig. 8. *Time dependence of the synthesis of extracellular proteins in ADPKD epithelial cells.* ^{35}S-methionine radiolabeled intracellular proteins (cells) were prepared as described in Figure 6 following the 24 hr incubation. At the times indicated 50 μl samples were analyzed for the extracellular ^{35}S-methionine radiolabeled proteins in the presence (+) or absence (−) of 5% FCS. Arrows on the right indicate the 220, 170 and 45 kDa extracellular proteins. (kDa, relative molecular weight)

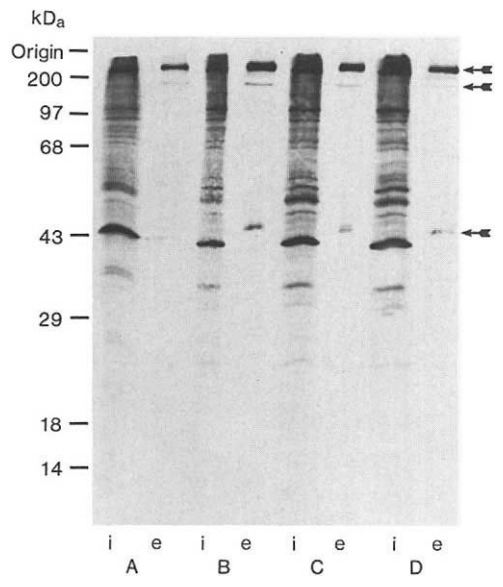

Fig. 9. *Intra- and extracellular proteins in ADPKD epithelial cell cultures from different patients.* ^{35}S-methionine radiolabeled intracellular (i) and extracellular (e) proteins were prepared as described in **Methods**, and separated by 12.5% acrylamide SDS-PAGE. Arrows on the right indicate the 220, 170 and 45 kDa extracellular proteins. (A, patient 1, primary cells; B, patient 2, passage 3; C, patient 3, passage 2; D, patient 4, passage 5; kDa, relative molecular weight)

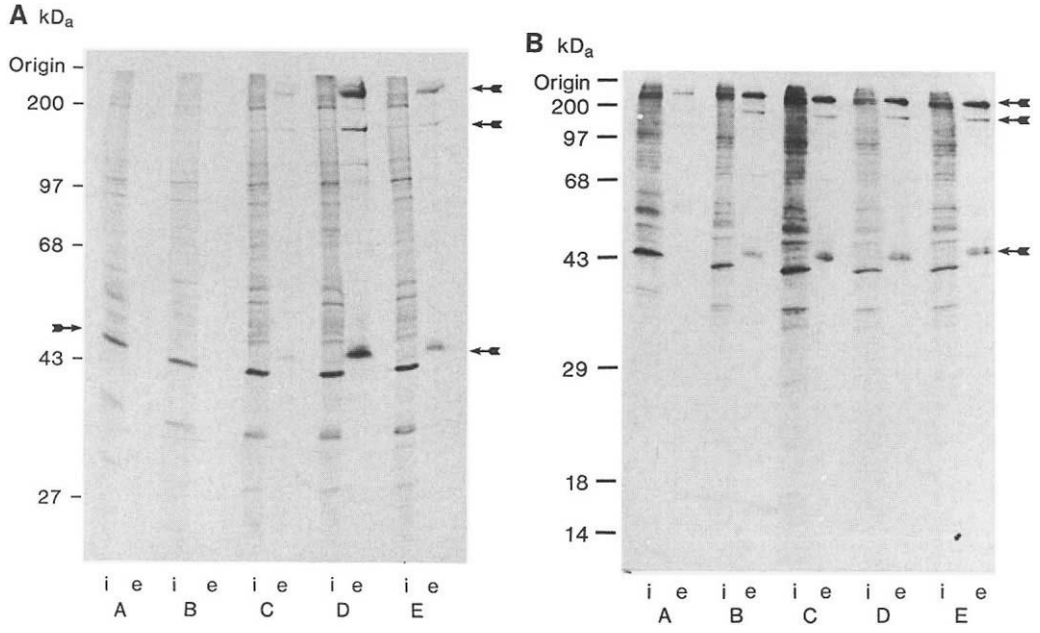

Fig. 10. *Intra- and extracellular proteins in different passages of HNC* (**A**) *and ADPKD* (**B**) *epithelial cells.* The ^{35}S-methionine radiolabeled intracellular (i) and extracellular (e) proteins were prepared and separated as described in **Methods**. The proteins in panel A were separated on 10% acrylamide gels and in panel B on 12.5% gels. Arrows on the right indicate the 220, 170 and 45 kDa extracellular proteins. The arrow on the left indicates the 42–44 kDa intracellular protein in HNC epithelial cells. (In each: Lane A, primary cells; Lane B, 1st passage; Lane C, 2nd passage; Lane D, 3rd passage; and Lane E, 5th passage; kDa, relative molecular weight)

be due to: 1) the HNC and ADPKD epithelia originating from different epithelial cell populations in the human kidney, or 2) the translation of the genetic information related to ADPKD causing primary or secondary effects that result in the altered biosynthesis of these proteins. Presently, neither of these possibilities can be excluded. In addition, the 2D gel electro-

phoresis used in these studies cannot accurately determine the absolute quantity of altered proteins in HNC and ADPKD cells. Proper quantitation of the specific proteins requires the use of specific protein ligands or antibodies to the biosynthetically altered proteins. Thus, further investigations are needed to examine the structure and possible function of these affected proteins.

In summary, this study establishes experimental procedures for a continuous HNC and ADPKD epithelial cell tissue culture system that can be used to overcome a critical deficit in material necessary for determination of the molecular and biochemical events related to the development of ADPKD. Using this system we have shown that altered protein synthesis occurs in ADPKD. The exact effect of this alteration on kidney function and structure in ADPKD must still be delineated.

Acknowledgments

This work was supported by a grant from the NIH DK34039. Portions of these studies were presented previously in abstract form at the Western Section of the Western Association of Physicians, 1988 [34], and the 20th annual meeting of the American Society of Nephrology, 1987 [35]. We thank S. Summer and H. Granot for their technical assistance, and L. Benson for her secretarial assistance, as well as the institutions and the many ADPKD patients that provided us with their end-stage kidneys. We also thank Molecular Dynamics for their assistance with the densitometric measurements of the 2D autoradiograms.

Reprint requests to Dr. Yosef Granot, C281, University of Colorado School of Medicine, 4200 E. 9th Avenue, Denver, Colorado 80262, USA.

References

1. GRANTHAM JJ: Polycystic kidney disease: A predominance of giant nephrons. *Am J Physiol* 224:F3–F10, 1983
2. GARDNER KD, EVAN AP: Polycystic kidney disease. An enigma evolves. *Am J Kidney Dis* 3:402–413, 1984
3. CARONE FA, KANWAR YS, BUTKOVSKI RJ: Tubular cell and basement membrane changes in polycystic kidney, in *Third International Symposium on Renal Basement Membrane*, edited by HUDSON B, PRICE R, London, Academic Press, 1987
4. VOLLMER WM, WAHL PW, BLAGG CR: Survival with dialysis and transplantation in patients with end-stage renal disease. *N Engl J Med* 308:1553–1558, 1983
5. BUTKOWSKI R, CARONE F, GRANTHAM J, HUDSON B: Tubular basement membrane changes in 2-amino-4,5-diphenylthiazole-induced polycystic disease. *Kidney Int* 28:744–751, 1985
6. KANWAR YS, CARONE FA: Reversible tubular cell and basement membrane changes in drug-induced renal cystic disease. *Kidney Int* 26:35–43, 1984
7. GRANTHAM JJ, GEISER JL, EVAN AP: Cyst formation and growth in autosomal dominant polycystic kidney disease. *Kidney Int* 31: 1145–1152, 1987
8. BERNSTEIN J, EVAN AP, GARDNER KD: Epithelia hyperplasia in human polycystic kidney diseases. *Am J Pathol* 129:92–101, 1987
9. GARDNER KD, EVAN AP, REED WP: Accelerated renal cyst development in reconditioned germ-free rats. *Kidney Int* 29:1116–1123, 1986
10. CUPPAGE FE, HUSEMAN RA, CHAPMAN A, GRANTHAM JJ: Ultrastructure and function of cysts from human adult polycystic kidneys. *Kidney Int* 18:375–381, 1980
11. EVAN AP, GARDNER KD, BERNSTEIN J: Polypoid and papillary epithelial hyperplasia. A potential cause of ductal obstruction in adult polycystic disease. *Kidney Int* 17:372–378, 1980
12. GRANTHAM JJ, GABOW PA: Polycystic renal disease, in *Diseases of the Kidney*, edited by SCHRIER RW, GOTTSHALK CW, Boston, Little Brown, 1988, pp. 583–615
13. CARONE FA, HOLENBERG PF, NAKAMURA S, PUNYARRIT P, GLOGOWSKI W, FLOURET G: Tubular basement membrane change occurs pari passu with the development of cyst formation. *Kidney Int* 35:1034–1040, 1989
14. GRANTHAM JJ, LEVINE E: Acquired cystic disease: Replacing one kidney disease with another. *Kidney Int* 28:99–105, 1985
15. TURANI H, LEVI J, ZEVIN D, KESSLER E: Acquired cystic disease and tumors in kidneys of hemodialysis patients. *Israel J Med Sci* 19:614–618, 1983
16. GREGOIRE JR, TORRES VE, HOLLEY KE, FARROW GN: Renal epithelial hyperplastic and neoplastic proliferation in autosomal dominant kidney disease. *Am J Kidney Dis* 9:27–38, 1987
17. COWLEY BD, SMARDO FL, GRANTHAM JJ, CALVET JP: Elevated c-myc protooncogene expression in autosomal recessive polycystic kidney disease. *Proc Natl Acad Sci USA* 84:8394–8398, 1987
18. REEDERS ST, BREUNING MH, DAVIES KE, NICHOLIS RD, JARMAN AP, HIGGS DR, PEARSON PL, WEATHERALL DJ: A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. *Nature* 217:542–544, 1985
19. REEDERS ST, BREUNING MH, CORNEY G, JEREMIAH SJ, MEERA R, KHAN P, DAVIES KE, HOPKINSON DA, PEARSON PL, WEATHERALL DJ: Two genetic markers closely linked to adult polycystic kidney disease on chromosome 16. *Brit Med J* 292:851–853, 1986
20. REEDERS ST, BREUNING MH, RYYNANEN MA, WRIGHT AF, DAVIES KE, KING AW, WATSON ML, WEATHERALL DJ: A study of genetic linkage heterogeneity in adult polycystic kidney disease. *Hum Genet* 76:3448–3451, 1987
21. KIMBERLING WJ, FAIN PR, KENYON BS, GOLDGAR D, SUJANSKY E, GABOW PA: Linkage heterogeneity of autosomal dominant polycystic kidney disease. *N Engl J Med* 319:913–918, 1988
22. ROMEO G, DEVATO M, COSTA G, RONCUZZI L, CATIZONE L, ZUCCHELLI P, GERMINO GG, KEITH T, WEATHERALL DJ, REEDERS ST: A second genetic locus for autosomal dominant polycystic kidney disease. *Lancet* 2:8–10, 1988
23. BRISSENDIN JE, ROSCOE JM, SILVERMAN M: Assessment of a linked DNA marker for presymptomatic diagnosis of adult polycystic kidney disease in North America. (abstract) *Kidney Int* 35:202, 1989
24. DAWSON DB, TORRES VE, CHARBONEAU JW, THIBODEAU SN: Detection of a family with autosomal dominant polycystic kidney disease loosely lined to DNA markers from 16p. (abstract) *Kidney Int* 35:203, 1989
25. WILSON PD, SCHRIER RW, BRECKON RD, GABOW PA: A new method for studying human polycystic kidney disease epithelia in culture. *Kidney Int* 30:371–378, 1986
26. STEINERT PM, STEVEN AC, ROOP DR: The molecular biology of intermediate filaments. *Cell* 42:411–419, 1985
27. CARDIFF RD, TAYLOR CR, WELLINGS SR, COLCHER D, SCHLOM J: Monoclonal antibodies in immunoenzyme studies of breast cancer. *Ann NY Acad Sci* 420:140–146, 1983
28. LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227:680–685, 1970
29. O'FARRELL PH: High resolution two-dimensional electrophoresis. *J Biol Chem* 250:4007–4021, 1975
30. CHAMBERLAIN JP: Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal Biochem* 98:132–136, 1979
31. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with Folin phenol reagent. *J Biol Chem* 193:265–273, 1951
32. KASSELBERG AG, ORTH DN, GRAY ME, STAHLMAN MT: Immunocytochemical localization of human epidermal growth factor/urogastrone in several human tissues. *J Histochem Cytochem* 33: 315–322, 1985
33. TSIVITSE P, ABBOUD HE, SAUNDERS C, KNAUSS TC: Effect of epidermal growth factor on cultured mesangial cells. (abstract) *Kidney Int* 31:184A, 1987
34. GRANOT Y, VAN PUTTEN V, SUMMER S, GRANOT H, GABOW P, SCHRIER RW: The biosynthesis of intra- and extracellular proteins by cultured human polycystic kidney cells. (abstract) *Clin Res* 36:135A, 1988
35. GRANOT Y, SUMMER S, VAN PUTTEN V, GRANOT H, GABOW P, SCHRIER RW: Establishment of a continuous tissue culture system from human polycystic kidney cells. (abstract) *Kidney Int* 33:191, 1987